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Therapeutic Compounds

This invention relates to a series of compounds derived which are derivatives of tricyclic lactam indoles and tricyclic lactam benzimidazoles and which inhibit poly (ADP-ribose) polymerase (PARP) and their use in the treatment of cancer, in particular breast cancer.

Homologous recombination (HR) has been shown to play an important role in repair of damage occurring at DNA replication forks in mammalian cells (2). Thus, cells deficient in HR show retarded growth and exhibit higher levels of genetic instability. It is believed that genetic instability due to loss of HR repair in human cancers significantly contributes to the development of cancer in these cells (1).

Post transcriptional modification of nuclear proteins by poly (ADP-ribosyl)ation in response to DNA strand breaks plays an important role in DNA repair, regulation of apoptosis, and maintenance of genomic stability.

Poly (ADP-ribose) polymerase (PARP-1) is the principal member of the PARP enzyme family and is an abundant nuclear protein in mammalian cells. PARP-1 catalyses the formation of poly (ADP-ribose) (PAR) polymers using NAD^+ as substrate. Upon DNA damage, PARP-1 binds rapidly to a DNA single-strand break (SSB) and catalyses the addition of negatively charged PAR chains to itself (automodification) and other proteins [see (3,4) for reviews]. The binding of PARP-1 to SSBs is believed to protect DNA lesions from further processing until PARP-1 is

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dissociated from the break by the accumulated negative charge resulting from PAR polymers (5, 6).

Although PARP-1 has been implicated in several nuclear processes, such as modulation of chromatin structure, DNA-replication, DNA repair and transcription, PARP-1 knockout mice develop normally (7). Cells isolated from these mice exhibit a hyper recombination phenotype and genetic instability in the form of increased levels of sister chromatic exchanges (SCE) micronuclei and tetraploidy (8, 10). Genetic instability may also occur in these PARP-1 knockout mice through telomere shortening, increased frequency of chromosome fusion and aneuploid (11), although all these results could not be repeated in another set of PARP-1 knockout mice (12). In the former mice knockout, PARP-1 null mutation rescued impaired V (D) J recombination in SCID mice (13).

These results support the view suggested by Lindahl and co-workers that PARP-1 has a protective role against recombination (5). It was proposed that binding of PARP-1 to ssDNA breaks prevents the recombination machinery from recognising and processing DNA lesions or, alternatively that the negative charges accumulated following poly (ADP-ribosyl)ation repel adjacent recombinogenic DNA sequences. Only the latter model is consistent with inhibition of PARP-1 itself and expression of a dominant negative mutant PARP-1, including SCE, gene amplification and homologous recombination (14-18).

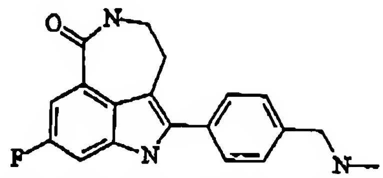
Studies based on treating cells with inhibitors of PARP-1 or cells derived from PARP-1 knockout mice indicate that the suppression of PARP-1 activity increases cell susceptibility to DNA damaging agents and inhibits strand break rejoining (3, 4, 8 – 11, 19, 20).

Inhibitors of PARP-1 activity have been used in combination with traditional cancer treatment regimes such as radio-therapy and chemotherapy (21). When the inhibitors were used in combination with methylating agents, topoisomerase poisons and ionising radiations they were found to enhance the effectiveness of these forms of treatment. However, such treatments are non-selective and as such cause damage and death to non-cancerous or 'healthy' cells. Furthermore, such treatments are known to give rise to unpleasant side effects.

Therefore, it is highly desirable to provide a treatment for cancer that is both effective and selective in the killing of cancer cells and which does not need to be administered in combination with radio-therapy or chemotherapy treatments.

Surprisingly it has been found that cells deficient in homologous recombination (HR) are hypersensitive to PARP inhibitors relative to wild type cells.

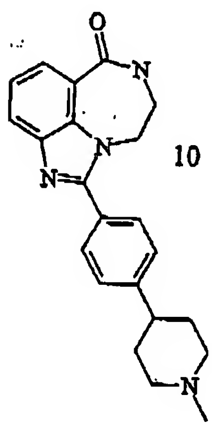
Thus, according to a first aspect of the present invention there is provided a compound for inhibiting the activity of PARP having formula I:



I

and pharmaceutically acceptable salts thereof.

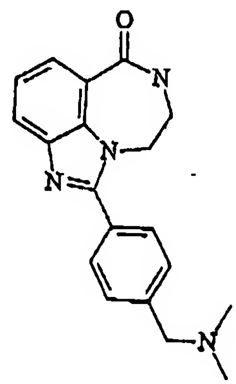
According to a second aspect of the present invention there is provided a compound for inhibiting the activity of PARP having formula II:



II

and pharmaceutically acceptable salts thereof.

According to a third aspect of the present invention there is provided a compound for inhibiting the activity of PARP having formula III:



III

and pharmaceutically acceptable salts thereof.

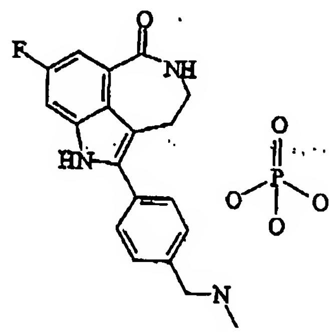
The compounds described herein can be prepared by synthetic routes based on those disclosed in WO 00/42040 and WO 01/16136.

It will be understood that where reference is made in this specification to compounds of formulas I to III the reference should be construed as extending also to their pharmaceutically acceptable salts and to other pharmaceutically acceptable bioprecursors (prodrug forms) where relevant. The term "prodrug" is used in the present specification to denote modified forms or derivatives of a pharmacologically active compound which biodegrade or are modified in vivo so as to become converted into said active compound after administration, especially oral or intravenous administration, in the course of therapeutic treatment of a mammal. Such prodrugs are commonly chosen because of an enhanced solubility in aqueous media which helps to overcome formulation problems, and also in some cases to give a relatively slow or controlled release of the active agent.

As referred to herein pharmaceutically acceptable salts include metal salts, phosphates and quaternary amines. The metal salts may be formed with alkali metals such as lithium, sodium or potassium.

Preferably, formula I, above, is administered in the form of a pharmaceutically acceptable phosphate salt having the follwing formula:

Formula I - phosphate



The present invention also relates to the therapeutic utility of the compounds described herein.

Thus, according to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula I, and pharmaceutically acceptable salts thereof, in the manufacture of a medicament.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula II, and pharmaceutically acceptable salts thereof, in the manufacture of a medicament.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula III, and pharmaceutically acceptable salts thereof, in the manufacture of a medicament.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula I, and pharmaceutically acceptable salts thereof, in the manufacture of a medicament for the treatment of a disease or condition that is caused by a genetic defect in a gene that mediates homologous recombination.

According to a further aspect of the present invention there is provided the use of a compound of formula II in the manufacture of a medicament for the treatment of a disease or condition that is caused by a genetic defect in a gene that mediates homologous recombination.

According to a further aspect of the present invention there is provided the use of a compound of formula III in the manufacture of a medicament for the treatment of a disease or condition that is caused by a genetic defect in a gene that mediates homologous recombination.

Diseases and conditions which are caused by a genetic defect in a gene that mediates homologous recombination include, but are not limited to cancer, in particular breast cancer.

As referred herein "cancer" or "tumour" includes, but is not limited to, cancer of the lung, colon, pancreas, stomach, ovary, cervix, breast, prostate bone, brain or skin.

The use of PARP inhibitors is particularly suitable in the treatment of cancer which is caused by a genetic defect in a gene wherein the said gene mediates homologous recombinations. Cancer cells of this type tend to be HR defective.

The specific sensitivity of HR defective tumours to PARP inhibition means that normally dividing "healthy" cells in patients which have adequate amounts of HR will be largely unaffected by the treatment.

A further advantage of treatment using PARP inhibitors is that the PARP inhibitors do not need to be administered as a combination therapy along with conventional radiotherapy or chemotherapy treatments thereby avoiding the side effects associated with these conventional forms of treatment.

A defect in a gene that mediates homologous recombination may be due to a mutation in, the absence of, or defective expression of, a gene encoding a protein involved in HR.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula I in the manufacture of a medicament for inducing apoptosis in HR defective cells.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula II in the manufacture of a medicament for inducing apoptosis in HR defective cells.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula III in the manufacture of a medicament for inducing apoptosis in HR defective cells.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula I in the manufacture of a medicament for the treatment of cancer.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula II in the manufacture of a medicament for the treatment of cancer.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula III in the manufacture of a medicament for the treatment of cancer.

Cancer cells suitable for treatment with the compounds described herein may be partially or totally deficient in HR. Preferably, the cells are totally deficient in HR.

The compounds described herein may be used to treat an inherited form of cancer wherein the patient to be treated has a familial predisposition to the cancer. However the said compounds are particularly suitable for the treatment of gene-linked hereditary cancer, and most particularly gene-linked hereditary breast cancer.

In a preferred aspect, the PARP inhibitor is useful in the treatment of cancer cells defective in the expression of a gene involved in HR. Genes with suggested function in HR include XRCC1, ADPRT (PARP-1), ADPRTL2, (PARP02) CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51, RAD51β, RAD51C, RAD51D, DMC1, XRCCR, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NB51, WRN, BLM KU70, RU80, ATM, ATR CHK1, CHK2, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, RAD1, RAD9 [See (2, 3, 5, 22-28) for reviews].

A gene involved in HR may be a tumour suppressor gene. The invention thus provides for the treatment of cancer cells defective in the expression of a tumour suppressor gene. Preferably, the tumour suppressor gene is BRCA1 or BRCA2.

Breast cancer is the most common type of cancer among women in the Western World. Certain families have a strong predisposition for breast cancer, which is often owing to an inherited mutation in one allele of either BRCA1 or BRCA2. However, one functional allele is maintained. Thus, individuals possessing the said mutation develop normally and have no phenotypic consequence from this mutation. However,

in one cell, the functional allele might be lost, making this cell cancerous and at the same time deficient in HR. This step is critical for the onset of a tumour (1).

Therefore, according to a still further aspect of the invention there is provided the use of a therapeutic amount of a compound of formula I in the manufacture of a medicament for the treatment of cancer cells defective in BRCA1 and/or BRCA2 expression.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula II in the manufacture of a medicament for the treatment of cancer cells defective in BRCA1 and/or BRCA2 expression.

According to a further aspect of the present invention there is provided the use of a therapeutic amount of a compound of formula III in the manufacture of a medicament for the treatment of cancer cells defective in BRCA1 and/or BRCA2 expression.

The cancer cells to be treated may bé partially or totally deficient in BRCA1 or BRCA2 expression. Suoh deficiencies can be identified using multiplex PCR techniques array techniques (29, 30) or using other screens known to the skilled person. Particularly useful techniques include real-time quantitiative RT-PCR, Northern blot, immumohistochemistry and Western Blot (31, 32).

Accordingly, the compounds of the present invention are of particular interest for the treatment of a range of selected cancer tumours, and the invention further provides a method for the treatment of a patient suffering from cancer.

The compounds described herein may be administered in a therapeutically effective non-toxic amount via any suitable route for effectively targeting cancer cells. Suitable administration routes include, but are not limited to, any of the following: oral, intravenous, intramuscular, intradermal, intranasal, or topical.

A therapeutically effective amount of the compounds described herein is typically one which is sufficient to achieve the desired effect and may vary according to the nature and severity of the disease condition, and the potency of the compound. It will be appreciated that different concentrations may be employed for prophylaxis than for treatment of an active disease.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01 to 50 mg/kg in mice and 0.01 mg/m^2 to 50 mg/m^2 body surface area in humans. Ultimately, however, the amount of active ingredient administered and the frequency of administration will be at the discretion of a physician.

Advantageously, only very low doses of PARP inhibiting compounds are needed to have a therapeutic effect in treating cancer thereby reducing systemic build up of the compounds and thus minimising any associated toxic effects.

While it may be possible for the compounds described herein to be administered alone as the 'raw' compound , it is preferable to present the compounds in a pharmaceutical composition.

All methods of formulation in making up such pharmaceutical compositions will generally include the step of bringing one of the compounds described herein into association with a carrier which constitutes one or more accessory ingredients. Usually, the formulations are prepared by uniformly and intimately bringing the compound of formula I into association with a liquid carrier or with a finely divided solid carrier or with both and then, if necessary, shaping the product into desired formulations.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of one of the compounds describe herein; as a powder or granules; or a suspension in an aqueous liquid or non-aqueous liquid such as a syrup, an elixir, an emulsion or a draught. Any one of the compounds described herein may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tables may be prepared by compressing, in a suitable machine, any one of the compounds described herein in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent,

surface active or dispersing agent. Moulded tables may be made by moulding, in a suitable machine, a mixture of any one of the powdered compound described herein with any suitable carrier.

A syrup may be made by adding any one of the compounds described herein to a concentrated, aqueous solution of a sugar, for example sucrose, to which may be added any desired accessory ingredient. Such accessory ingredient(s) may include flavourings, an agent to retard crystallisation of the sugar or an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol.

Formulations for rectal administration may be presented as a suppository with a usual carrier such as cocoa butter.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of any one of the compounds describe herein which is preferably isotonic with the blood for the recipient.

In addition to the aforementioned ingredients, formulations of this invention, for example ointments, creams and the like, may include one or more accessory ingredients, for example a diluent, buffer, flavouring agent, binder, surface active agent, thickener, lubricant and/or a preservative (including an antioxidant) or other pharmaceutically inert excipient.

The compounds of this invention may also be made up for administration in liposomal formulations which can be prepared by methods well-known in the art.

Thus, according to a further aspect of the present invention there is provided a pharmaceutical composition comprising a compound of formula I, or pharmaceutically acceptable salt thereof, as an active agent.

According to a further aspect of the present invention there is provided a pharmaceutical composition comprising a compound of formula II, or pharmaceutically acceptable salt thereof, as an active agent.

According to a further aspect of the present invention there is provided a pharmaceutical composition comprising a compound of formula III, or pharmaceutically acceptable salt thereof, as an active agent.

The pharmaceutical composition may further comprise at least one other ingredient providing a compatible pharmaceutically acceptable additive, carrier diluent carrier or excipient and may be presented in unit dosage form.

The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not deterious to the recipient thereof.

The possible formulations include those suitable for oral, rectal, topical and parenteral (including subcutaneous, intramuscular and intravenous) administration or for administration to the lung or another absorptive site such as the nasal passages.

The compounds referred to herein may be administered in combination with other anti-cancer compounds.

The present invention also includes a method of treating cancer in mammals by administering the compounds described herein and their pharmaceutically acceptable salts.

Thus, according to a further aspect of the present invention there is provided a method for the treatment of cancer in mammals comprising administering a compound of formula I, or a pharmaceutically acceptable salt thereof.

Thus, according to a further aspect of the present invention there is provided a method for the treatment of cancer in mammals comprising administering a compound of formula II, or a pharmaceutically acceptable salt thereof.

Thus, according to a further aspect of the present invention there is provided a method for the treatment of cancer in mammals comprising administering a compound of formula III, or a pharmaceutically acceptable salt thereof.

The present invention will now be described by way of example only with reference to the accompanying figures wherein:

Figure 1 is a graph showing cell survival in the presence of PARP inhibitor of formula III in AA8 cell line, IrsISF cell line and CxR3 cell line;

Figure 2 is a graph showing cell survival in the presence of PARP inhibitor of formula III in V79 cell line, VC8 cell line and VC8B2 cell line;

Figure 3 is a graph showing cell survival in the presence of PARP inhibitor of formula I in V79 cell line, VC8 cell line and VC8B2 cell line;

Figure 4 is a bar chart showing PARP activity in VC8; V79, VC8#13 and VC8, VC8#13 and VC8+B2 cell lines in the presence of PARP inhibitor of formula III;

Figure 5 is a pair of graphs showing inhibition of cellular PARP activity in the presence of PARP inhibitor of formula I and III in permeabilised (upper graph) and intact (lower graph) L1210 cells;

Figure 6 is a pair of bar charts showing the blood and tumour pharmacokinetics and pharmacodynamics with formula I-phosphate at 1 mg/kg (upper) and 10 mg/kg (lower) in mice bearing SW620 xenografts;

Figure 7 is a bar chart showing the pharmacokinetics and pharmacodynanics with formula III in mice bearing SW620 xenografts;

Figure 8 is a graph showing the tumour growth (median relative tumour volume) in mice bearing SW620 xenografts following treatment with formula III in combination with temozolomide (TMZ) and with TMZ alone;

Figure 9 is a graph showing the tumour growth (median relative tumour volume) of mice bearing SW620 xenografts following treatment with formula I-phosphate in combination with temozolomide (TMZ) and with formula I-phosphate and TMZ alone;

Fig. 1 shows the percentage survival of AA8, IrS ISF and CxR3 cell lines when treated with various concentrations of the compound of formula III. Formula III was found to be most active against IrS ISF, which lacks XRCC3, having an LC_{50} (the concentration of the active component that kills 50% of the cells) of 100 nM.

Fig. 2 shows the percentage survival of V79-Z, VC8 and VC8B2 cell lines when treated with various concentrations of the compound of formula III. Formula III was found to be most effective against the VC8 cell line, which lacks BRCA2, having an LC_{50} value of 43 nM and an LC_{90} (concentration of active component that kills 90% of the cells) was 1200 nM.

Fig. 3 shows the percentage survival of V79-Z, VC8 and VC8B2 cell lines when treated with various concentrations of the compound of formula I. Formula I was found to be most effective against the VC8 cell line, which lacks BRCA2, having an LC_{50} value of 12 nM, LC_{90} was 27 nM

Fig. 4 shows PARP activity of various cell lines when treated with various concentrations of the compound of formula III. The graph of Fig. 3 is divided into four result sets for each respective cell line. The first bar of each set shows the background PARP activity (no oligo present, so PARP activity is dependent upon endogenous DNA breaks), the second bar is total stimulatable (by oligo) PARP activity and the third and fourth bars show the PARP activity in the presence of the compound of formula III.

Fig. 5 shows the effect of Compounds Formula I and III on PARP activity.

Cells used to obtain the results shown in Fig. 5 were either permeabilised with digitonin and then assayed for total stimulatable (by oligo) PARP activity in the presence and absence of PARP inhibitor of formula I and formula III or exposed to one of said PARP inhibitors for 20 minutes prior to permeabilisation and assayed for total stimulatable PARP activity.

There was no difference in the PARP inhibitory activity of the compounds of formula I and formula III when the cells were permeabilised prior to adding the inhibitor

compound but the compound of formula I was more potent in intact cells, possibly because it accumulates within cells to a higher degree.

Fig. 6 shows the plasma and tumour concentrations of the compound of formula I, and its pharmacokinetic effect on mouse peripheral blood lymphocytes (pbl parp) and SW620 xenografts (tumour PARP), at various times following intraperitoneal administration of the phosphate salt of compound of formula I. The phosphate salt of the compound of formula I increases the solubility of formula I. However, on administration to an animal (including human) plasma phosphatases break the phosphate salt of formula I (formula I-phosphate) down to the parent compound i.e. formula I.

It is evident form fig. 6 that thirty minutes after administration of formula I-phosphate at 10 mg/kg highlevels of the parent compound were detected in both plasma and tumour. The concentration of formula 1 decreased with time more rapidly in the plasma than in the tumour and at 24 hr after administration significant levels were detectable in the tumour but none could be detected in the plasma. There was a profound and sustained inhibition of PARP activity in both pbls and tumour: <50% control up to 24 hr.

After administration of formula I-phosphate at 1 mg/kg lower levels of the compound of formula I can be found in both the plasma and tumour and consequently there was a less pronounced effect on PARP activity.

Fig. 7 shows the plasma and tumour concentrations of the compound of formula III, and its pharmacokinetic effect on SW620 xenografts (tumour PARP act), at various times following intraperitoneal administration of the 10 mg/kg of compound of formula III. This compound also distributes well to the tumour and is preferentially retained with time and similarly inhibits PARP activity in the tumour.

Fig. 8 shows that for 20 days from administration of temolozomide (68 mg/kg daily x5) the tumour xenograft has progressively reduced in size. However, shortly after this time the tumour size begins to increase. When a compound of formula III (5 mg/kg daily x 5) is administered in conjunction with temozolomide the tumour shrinks significantly for around 15 days, to an undetectable size, the tumour size remains undetectable for a further 50 days thereafter when it begins to increase in size. When a larger dose of formula III (15 mg/kg daily x 5) is administered the tumour size remains undetectable for a further 80 days until the end of the experiment when no tumour was detectable at autopsy i.e. complete tumour regression.

Fig.9 shows a similar pattern to that seen in fig. 8 following the administration of formula I-phosphate (at 0.1 mg/kg and 1.0 mg/kg) in combination with temolozomide.

Table 1. Genotype and origin of cell lines used in this study.

Cell line	Genotype	Defect	Origin	Reference	Comments
AA8	wt	wt	CHO	[41]	Chinese hamster ovary cell line
irs1SF	*$XRCC3^-$*	$XRCC3^-$, deficient in HR	AA8	[41]	Radiation-sensitive cell line derived from AA8 which lacks XRCC3 a component of HR pathway
CXR3	*XRCC3-* + hXRCC3	wt	irs1SF	[41]	irs1SF transfected with hXRCC3 gene
V79-Z	wt	wt	V79	[42]	V79 are hamster lung fibroblasts
VC8	*BRCA2-*	$BRCA2^-$, deficient in HR	V79-Z	[42]	VC8 are radiation sensitive derivatives of V79 which are deficient in BRCA2
VC8#13	*$BRCA2^-$* +*hBRCA2*	wt	VC8	[42]	VC8 with chromosome 13 containing *hBRCA2*
VC8+B2	*$BRCA2^-$* +*hBRCA2*	wt	VC8	[42]	VC8 transfected with *hBRCA2*

Materials and Methods

Cytotoxicity of PARP inhibitors to cells deficient in HR (XRCC3 or BRCA2)

Cell culture

The AA8, irs1SF and CXR3 cell lines were provided by Larry Thompson [41].

The VC-8, VC-8+B2, VC-8#13 were a gift from Malgorzata Zdienicka [42]. All cell lines in this study were grown in Dulbecco's modified Eagle's Medium (DMEM) with 10% Foetalbovine serum and penicillin (100 U/ml) and steptomycin sulphate (100 μg/mL) at 37°C under an atmosphere containing 5% CO_2.

Toxicity assay – clonogenic survival assay

Exponentially growing cells in 6-well plates were exposed to the compound of formula III at the concentrations indicated in Fig 2 in 1% DMSO or 1% DMSO alone in medium for 24 hours.

The cells were harvested by trypsinisation, counted and seeded at varying densities in 10 cm dishes in fresh medium in the absence of drug for colony formation.

7-10 days later the dishes were fixed with methanol:acetic acid 3:1 and stained with 0.4% crystal violet.

Colonies were counted and the survival relative to 1%DMSO control treated cells calculated.

PARP activity assay

Exponentially growing cells were exposed to 1% DMSO in culture medium (control) or a compound of formula I or III in 1% DMSO at the concentrations indicated in Fig. 4 to cells permeabilised with digitonin, or intact cells for 20 minutes prior to washing and digitonin-permeabilization. PARP activity was measured by incorporation of a

[32P] labelled NAD+ substrate into TCA precipitateble polymers after stimulation by the addition of a blunt-ended oligonucleotide and compared with non-oligonucleotide-stimulated cells. PARP activity in tumour homogenates (1 in 40 in isotonic buffer) from formula III-treated mice was measured in the same way. PARP activity in pbls and tumour homogenates from fomula I-phospate treated mice was measured by immunological detection of polymer using the 10H antibody. Briefly, tumour homogenates diluted to up to 1:1000 in isotonic buffer were incubated with 350 μM NAD for 6 min and blotted onto nitrocellulose membrane. The poly(ADP-ribose) (PAR) polymer formation was quantified by chemiluminescence detection using a Fuji LAS3000 UV Illuminator by reference to serial dilutions of a PAR standard, following incubation with 10H antibody to PAR and a secondary anti-mouse antibody. The results were standardised by reference to the measured protein content of the homogenate.

It is of course to be understood that the invention is not intended to be restricted to the details of the above embodiments which are described by way of example only.

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